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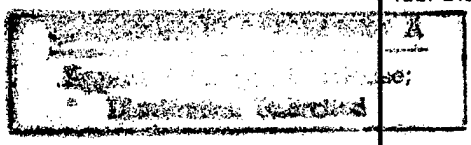

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13. ABSTRACT *(Maximum 200 words)*

Novel plasmid-like DNA from various marine green algae in the orders Siphonocladales and Cladophorales was isolated and characterized to determine its ultimate utility as vectors for introducing foreign/modified DNA into green plant cells. Although the molecules are linear in nature, they are single DNA strands folded back on themselves by intramolecular base pairing. They are extrachromosomal DNAs which seem to replicate autonomously. Sequencing cloned restriction fragments reveals putative open reading frames that encode portions of psb and psa genes - whose products are essential components of photosystems II and I of photosynthesis. These molecules appear to be transcriptionally active, labeling transcripts large enough to encode the proteins. The plasmids are localized within each chloroplast. Thus, these molecules probably evolved from chloroplast DNA. The collective features of these DNAs make them potentially amenable to development as vectors for transforming cells or chloroplasts of these and other green plants.

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OBJECTIVES: To determine the fundamental characteristics and utility of novel extrachromosomal DNA in coenocytic/multinucleate marine green algae. This includes determining: 1) its complexity and what genes it encodes, 2) whether it is self-replicating DNA or chromosomal in origin, 3) its occurrence among different stages/isolates and the presence of homologous DNA in related genera of green algae, 4) its expression patterns, 5) its subcellular location, and 6) its utility as vectors for introducing foreign genes into algae and other green plants.

APPROACH: Clones of this novel DNA were sequenced and analyzed for evidence of open reading frames and other hallmarks. The clones also served as probes for Southern/northern analysis of DNA/RNA from various strains and genera of coenocytic green algae, and for in situ hybridization. Electron microscopy was utilized to determine whether the DNA is circular and/or linear in nature. Homologous and heterologous tranformation will investigate the ability of constructs of algal and reporter DNA to replicate and be expressed natively and in foreign cells.

ACCOMPLISHMENTS DNA extracted from all five isolates of Ernodesmis, from other members of the Siphonocladales (Boergesenia, Ventricaria, Valoniopsis, Cladophoropsis, Valonia and Chamaedoris), and from Chaetomorpha (Cladophorales), contains low molecular weight (LMW) DNA ranging from 1.5 to 3.0 kb in size. Such DNA is absent in Chlamydomonas, Nitella, Microdictyon and Caulerpa. It is clear from electrophoresis gels that different genera have differing degrees of complexity of LMW DNA: some have a single broad band of DNA, while others have several sharper bands. We have concentrated efforts on the 2.2 kb DNA from the San Salvador isolate of Ernodesmis, partly because we had a set of cloned HinFI restriction fragments of this DNA in hand. These 22 clones are termed the "A" (1.2 kb), "B" (0.9 kb) and "C" (0.7 kb) clones. We hybridized Southern blots of total Ernodesmis DNA with probes made from each of the "ABC" clones, and 21 of the 22 labeled only the 2.2 kb DNA. [Putative "full length" (2.2 kb) clones turned out to be fragments of chromosomal DNA with no homology to the 2.2 kb DNA.] Heterologous probes from Chlamydomonas indicate that nuclear and chloroplast genomes are contained within the high molecular-weight chromosomal DNA, as expected. This indicates that the LMW DNA lacks any homology to chromosomal DNA, which is direct evidence that the 2.2 kb DNA is not a mere degradation product created during extraction, but

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that it is truly extrachromosomal. This also suggests that the 2.2 kb DNA may be replicating autonomously from chromosomal DNA. Some of these "ABC" probes cross-hybridize with LMW DNA from the Puerto Rican isolate, less so with DNA from the Barbadian and Jamaican isolates, and not at all with the Mexican isolate from the Gulf of California, under conditions of high stringency. At more moderate stringency some clones will cross-hybridize to the Mexican isolate as well as to LMW DNA from a few of the related genera (Boergesenia).

The LMW DNA from Ernodesmis has plasmid-like features: it rapidly reanneals after heat-denaturing/snap-cooling, and it is greatly enriched when we extract cells with a modified alkaline-lysis protocol. This DNA is apparently retained by unicellular/uninucleate reproductive stages, since its presence is maintained in cultures derived from asexual zoospores. Preliminary data extracting DNA from isolated chloroplasts reveal that the 2.2 kb DNA is greatly enriched in this fraction, suggesting that it may be located in chloroplasts. Use of a few of the cloned DNAs as probes for fluorescence in situ hybridization verified that indeed these molecules are exclusively localized within the chloroplasts of both Ernodesmis and Ventricaria. They appear as punctate spots in a spherical arrangement around the prominent pyrenoid occurring in each chloroplast. This would explain how the molecules are transmitted through uninucleate stages in the life cycles of these coenocytic (multinucleate without internal cross walls) algae - i.e., via the chloroplast in each zoospore.

Sequence data show that the 2.2 kb DNA in Ernodesmis is a population of heterogeneous molecules. The complete nucleotide sequences of all 21 clones of HinFI fragments of this DNA have been determined and analyzed, verifying that it comprises a population of heterogeneous molecules in vivo. Southern blotting data support this: some probes differentially label two or more bands within the 2.2 kb band. Also, when the DNA is restricted with multiple enzymes ("restriction fragment length polymorphism" or RFLP), differential labeling is found with most of the different probes. A comprehensive analysis verifies that our clones must be part of at least 11-13 different native DNA molecules. This level of heterogeneity, which exceeds that of all known plasmid-containing algae and is novel among eukaryotes, is quite surprising given the uniform size of these molecules. We hypothesize that replication of the extrachromosomal DNAs may be tightly controlled, perhaps by common terminal sequences that have yet to be identified.

Sequencing also shows that most clones have one or multiple tandem- and/or direct-repeating sequences, none of which are shared among the different clones. Repeated sequences are often hallmarks of linear plasmids. Four of the 17 unique clones contain open reading frames (ORFs) with high levels of identity to portions of four different photosynthesis-related (psb and psa) genes. These photosynthesis-related sequences represent the only identifiable genes found so far on the plasmid-like DNAs in these algae. Collectively these data indicate that the plasmid-like DNAs here are chloroplastidic in location and probably in evolutionary origin as well. Since the plasmids do not hybridize to chloroplast chromosomal DNA in Southern blots, it is postulated that either the plasmid sequences have diverged greatly from chromosomal DNA over evolutionary time or that at least some of the psb/a genes occur solely in the plasmd DNA. We also cloned a series of BamHI restriction fragments of the 2.8 kb extrachromosomal DNA from Ventricaria for comparative purposes. These DNAs possess all the abovementioned features.

All of the "ABC" probes tested label a 4.4 kb band in northern blots of total RNA, and six label additional bands ranging from 0.25 to 2.3 kb. The 4.4 kb band is contaminating DNA in our RNA preparations because it can be eliminated by pre-treating the RNA sample with RNase-free DNase I. Also, if the 2.2 kb DNA is denatured and run on denaturing gels, it does indeed migrate at 4.4 kb. This striking finding implies that each molecule of the the 2.2 kb DNA is actually a single strand of DNA folded back on itself in the native state! This corroborates the ultrastructural data. In the electron microscope, extrachromosomal DNA from the three different organisms examined (Ernodesmis, Boergesenia and Ventricaria) appears to comprise only linear molecules. In Ernodesmis, a small percentage (5-10%) of the molecules have a visible "loop" at one end. Therefore, we believe that this DNA is predominantly a long inverted repeat (resulting in it folding back on itself) with a central unique region that would result in the loop of unpaired sequences. The size of this unique region would determine the size of the loop. If it is very small, it would not be visible in the electron microscope, as appears to be the case in most molecules. The presence of a loop at minimally one end of each molecule explains the problems encountered in cloning directly entire molecules.

The smaller bands in northern blots of Ernodesmis RNA do appear to be RNA transcripts: they are rapidly digested by RNase A. The four clones with recognizeable ORFs in them also label RNA transcripts in northern blots. [A fifth one labeling a 2.2-2.3 kb band has no recognizeable ORFs in its sequence, perhaps representing some novel gene(s).] The sizes of the transcripts labeled are sufficient to encode the psb (psbB, psbC, psbF) or psa (psaB) proteins indicated by their ORFs. In one case, we have verified that the ORF sequence itself is responsible for the clone's hybridization in northern blots. One of the clones also apparently hybridizes to an authentic psbB transcript in northerns, since the same band labels with a heterologous psbB probe from Chlamydomonas. Of the Ventricaria clones examined so far, four of them label one or more transcripts ranging from 0.25 to 4.5 kb in size. One of them contains a psbA-like ORF. There is evidence that there may be multiple conformations or multimers of the LMW DNA in Ventricaria, unlike the case in Ernodesmis. Although we cannot yet be certain as to the exact origin of the psb/psa transcripts, it would appear that the extrachromosomal DNA is transcriptionally active. This finding gives the molecules biological relevance. The discovery of portions of genes encoding photosynthesis-related proteins combined with the localization of this plasmid-like DNA in chloroplasts, supports the notion that these molecules may have physiological significance in photosynthesis.

SIGNIFICANCE: If indeed the psb/psa transcripts are found to originate from the chloroplast plasmids instead of chromosomal DNA, this would have broad biological interest by being unique among all known photosynthetic plants. In all other known cases, the psa/b proteins are encoded within the chloroplast chromosomal DNA. Might these coenocytic green algae represent an early stage in the evolution of chloroplast DNA? The indication that these plasmids are transcriptionally active suggests that other genes incorporated into these molecules might similarly be transcribed. Combined with the indication that these DNAs replicate autonomously, it would seem that the plasmids from these green algae may indeed be useful for developing vectors for introducing foreign or modified genes into these and other green plant cells. Given their apparent function in encoding genes for photosynthesis and their location in chloroplasts, they potentially could be developed into the

first vectors for transforming chloroplasts as well. The presence of genes putatively encoding photosynthetic proteins further suggests that these DNAs may be useful for manipulating the process of photosynthesis and perhaps other aspects of plant growth and development as well. Applying plasmid-based transformation technology to transforming higher green-plant cells and/or their chloroplasts could revolutionize the way transformation is currently carried out, greatly improving the efficiency and facility of higher-plant transformation.

WORK PLAN (IMMEDIATE FUTURE): We recently isolated many near-full-length clones of Ernodesmis and Ventricaria plasmid DNA, after blunt-ending molecules with mung bean nuclease. We are beginning to sequence and analyze them. Work is also underway to create chimeric constructs containing portions of the Ernodesmis plasmid DNA with antibiotic resistance genes. We are attempting to transform E. coli cells with these chimeras to determine empirically where the origins of replication are located. Finally, we are trying to determine the source of the psb/psa transcripts with the ribonuclease protection assay and by isolating and sequencing cDNAs from an Ernodesmis cDNA library.

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